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(54) Title: FRUIT-SPECIFIC TRANSCRIPTIONAL FACTORS (57) Abstract Fruit-specific regulatory regions are identified employing cDNA screening. The resulting fruit-specific regulatory regions are manipulated for use with foreign sequences for introduction into plant cells to provide transformed plants having fruit with a modified phenotypic property. The invention is exemplified with a tomato fruit-specific promoter which is active throughout the stages of fruit ripening.		

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FRUIT-SPECIFIC
TRANSCRIPTIONAL FACTORS

5

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Application Serial No. 168,190, filed March 15, 1988, which is a continuation-in-part of Application Serial
10 No. 054,369 filed May 26, 1987, which applications are incorporated herein by reference.

INTRODUCTION

Technical Field

15 This invention relates to DNA expression cassettes capable of directing fruit-specific expression of in vitro constructed expression cassettes in plants. The invention is exemplified by promoters useful in fruit-specific transcription in a tomato plant.

20

Background

Manipulation of plants has proven to be significantly more difficult than manipulation of prokaryotes and mammalian hosts. As compared to
25 prokaryotes and mammalian cells, much less was known about the biochemistry and cell biology of plant cells and plants. The ability to transform plant cells and regenerate plants is unique to flora since other differentiated species provide readily available trans-
30 formable germ cells which may be fertilized and introduced into the live host for fetal development to a mature fetus. There has been substantial interest in modifying the ovum with inducible transcriptional initiation regions to afford inducible transcription and
35 expression of the gene introduced into the ovum, rather than constitutive expression which would result in expression throughout the fetus.

Also, for plants, it is frequently desirable to be able to control expression at a particular stage in the growth of the plant or in a particular plant part. During the various stages of the growth of the plant, and as to the various components of the plant, it will frequently be desirable to direct the effect of the construct introduced into the entire plant or a particular part and/or to a particular stage of differentiation of the plant cell. For this purpose, regulatory sequences are required which afford the desired initiation of transcription in the appropriate cell types and/or at the appropriate time in the plant development, without having serious detrimental effects on the plant development and productivity.

It is therefore important to be able to isolate sequences which can be manipulated to provide the desired regulation of transcription in a plant cell host during the growing cycle of the plant. One aspect of this interest is the ability to change the phenotype of fruit, so as to provide fruit which will have improved aspects for storage, handling, cooking, organoleptic properties, freezing, nutritional value, and the like.

Relevant Literature

cDNA clones from tomato displaying differential expression during fruit development have been isolated and characterized (Mansson et al., Mol. Gen. Genet. (1985) 200:356-361; Slater et al., Plant Mol. Biol. (1985) 5:137-147). The studies have focused primarily on mRNAs which accumulate during fruit ripening. One of the proteins encoded by the ripening-specific cDNAs has been identified as polygalacturonase (Slater et al., Plant Mol. Biol. (1985) 5:137-147). A cDNA clone which encodes tomato polygalacturonase has been sequenced. Grierson et al., Nucleic Acids Research (1986) 14:8395-8603. The concentration of polygalac-

turonase mRNA increases 2000-fold between the immature-green and red-ripe stages of fruit development. This suggests that expression of the enzyme is regulated by the specific mRNA concentration which in turn is regulated by an increase in transcription. Della Penna et al., Proc. Natl. Acad. Sci. USA (1986) 83:6420-6424. Mature plastid mRNA for psbA (one of the components of photosystem II) reaches its highest level late in fruit development, whereas after the onset of ripening, plastid mRNAs for other components of photosystem I and II decline to nondetectable levels in chromoplasts. Piechulla et al., Plant Mol. Biol. (1986) 7:367-376.

Other studies have focused on cDNAs encoding genes under inducible regulation, e.g. proteinase inhibitors which are expressed in response to wounding in tomato (Graham et al., J. Biol. Chem. (1985) 260:6555-6560; Graham et al., J. Biol. Chem. (1985) 260:6561-6564) and on mRNAs correlated with ethylene synthesis in ripening fruit and leaves after wounding. Smith et al., Planta (1986) 168:94-100.

Leaf disc transformation of cultivated tomato is described by McCormick, et al., Plant Cell Reports (1986) 5:81-89.

SUMMARY OF THE INVENTION

Novel DNA constructions are provided employing a "fruit-specific promoter," particularly those active beginning at or shortly after anthesis or beginning at the breaker stage, joined to a DNA sequence of interest and a transcriptional termination region. A DNA construct may be introduced into a plant cell host for integration into the genome and transcription regulated at a time at or subsequent to anthesis. In this manner, high levels of RNA and, as appropriate, polypeptides, may be achieved during formation and/or ripening of fruit.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of the cDNA clones pCGN1299 (2A11) and pCGN1298 (3H11). The amino acid sequence of the polypeptide encoded by the open reading frame is also indicated.

Figure 2 is a comparison of 2A11 to pea storage proteins and other abundant storage proteins:

(a) 2A11 (residues 33-46) is compared to PA1b and the reactive site sequences of some protease inhibitors, PA1b (residues 6-23), chick pea inhibitor (residues 11-23), lima bean inhibitor (residues 23-35), human α -antitrypsin reactive site peptide. The arrow indicates the reactive site.

(b) is a comparison of the amino terminal sequence of 2A11 with the amino termini of a range of seed proteins. The data have been modified or deletions introduced to maximize homology; conserved residues are shown boxed. The sequences are from the following sources: PA1b; barley chloroform/methanol-soluble protein d; wheat albumin; wheat α -amylase inhibitor 0.28; millet bi-functional inhibitor; castor bean 2S small subunit; and napin small subunit.

Figure 3 is a schematic diagram of the construction of the binary plasmid pCGN783; (a) through (f) refer to the plasmid constructions in Example 6.1.

Figure 4 shows the complete sequence of the 2A11 genomic DNA cloned into pCGN1273 from the XhoI site (position 1 at the 5' end) to the EcoRI site (position 4654).

Figure 5 shows the nucleotide sequence of a polygalacturonase (PG) genomic clone.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, DNA constructs are provided which allow for modification of plant phenotype during fruit maturation and ripening. The DNA constructs provide for a regulated transcrip-

tional initiation region associated with fruit development and ripening. Downstream from and under the transcriptional initiation regulation of the fruit related initiation region will be a sequence of interest which will provide for modification of the phenotype of the fruit. Desirably, integration constructs may be prepared which allow for integration of the transcriptional cassette into the genome of a plant host. Conveniently, the vector may include a multiple cloning site downstream from the fruit related transcriptional initiation region, so that the integration construct may be employed for a variety of sequences in an efficient manner.

Of particular interest is a transcriptional initiation region which is activated at or shortly after anthesis, so that in the early development of the fruit, it provides the desired level of transcription of the sequence of interest. Normally, the sequence of interest will be involved in affecting the process in the early formation of the fruit or providing a property which is desirable during the growing (expansion) period of the fruit, or at or after harvesting.

The ripening stages of the tomato may be broken down into mature green, breaker, turning, pink, light red and red. Desirably, the transcriptional initiation region maintains its activity during the expansion and maturation of the green fruit, more desirably continues active through the ripening or red fruit period. Comparable periods for other fruit are referred to as stages of ripening. The invention is not limited to those transcriptional initiation regions which are activated at or shortly after anthesis but also includes transcriptional initiation regions which are activated at any of the ripening stages of the fruit.

The transcriptional initiation region may be native or homologous to the host or foreign or heterologous to the host. By foreign is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. Of particular interest is a tomato fruit-specific transcriptional initiation region referred to as 2A11 which regulates the expression of a 2A11 cDNA sequence described in the Experimental section. The 2A11 transcriptional initiation region provides for an abundant messenger, being activated at or shortly after anthesis and remaining active until the red fruit stage. The expressed protein is a sulfur-rich protein similar to other plant storage proteins in sulfur content and size. Also of interest is the transcriptional initiation region which regulates expression of the enzyme polygalacturonase, an enzyme which plays an important role in fruit ripening. The polygalacturonase promoter is active in at least the breaker through red fruit stage.

Other fruit-specific promoters may be activated at times subsequent to anthesis, such as prior to or during the green fruit stage, during pre-ripe (e.g., breaker) or even into the red fruit stage.

A transcriptional initiation region may be employed for varying the phenotype of the fruit. Various changes in phenotype are of interest. These changes may include up- or down-regulation of formation of a particular saccharide, involving mono- or polysaccharides, involving such enzymes as polygalacturonase, levansucrase, dextranucrase, invertase, etc.; enhanced lycopene biosynthesis; cytokinin and monellin synthesis. Other properties of interest for modification include response to stress, organisms, herbicides, bruising, mechanical agitation, etc., change in growth regulators, organoleptic properties, etc. For antisense or complementary sequence transcription, the sequence will

usually be at least 12, more usually at least 16 nt. Antisense sequences of interest include those of polygalacturonase, sucrase synthase and invertase.

5 The transcriptional cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region, a sequence of interest, and a transcriptional and translational termination region functional in plants. One or more introns may be also be present. The DNA sequence may
10 have any open reading frame encoding a peptide of interest, e.g. an enzyme, or a sequence complementary to a genomic sequence, where the genomic sequence may be an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, e.g. splicing, or translation.
15 The DNA sequence of interest may be synthetic, naturally derived, or combinations thereof. Depending upon the nature of the DNA sequence of interest, it may be
20 desirable to synthesize the sequence with plant preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest.

25 In preparing the transcription cassette, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed for
30 joining the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. Toward this end, in vitro mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like may be employed, where
35 insertions, deletions or substitutions, e.g. transitions and transversions, may be involved.

The termination region which is employed will be primarily one of convenience, since the termination regions appear to be relatively interchangeable. The termination region may be native with the transcriptional initiation region, may be native with the DNA sequence of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid of A. tumefaciens, such as the octopine synthase and nopaline synthase termination regions.

By appropriate manipulations, such as restriction, chewing back or filling in overhangs to provide blunt ends, ligation of linkers, or the like, complementary ends of the fragments can be provided for joining and ligation.

In carrying out the various steps, cloning is employed, so as to amplify the amount of DNA and to allow for analyzing the DNA to ensure that the operations have occurred in proper manner. A wide variety of cloning vectors are available, where the cloning vector includes a replication system functional in E. coli and a marker which allows for selection of the transformed cells. Illustrative vectors include pBR332, pUC series, M13mp series, pACYC184, etc. Thus, the sequence may be inserted into the vector at an appropriate restriction site(s), the resulting plasmid used to transform the E. coli host, the E. coli grown in an appropriate nutrient medium and the cells harvested and lysed and the plasmid recovered. Analysis may involve sequence analysis, restriction analysis, electrophoresis, or the like. After each manipulation the DNA sequence to be used in the final construct may be restricted and joined to the next sequence, where each of the partial constructs may be cloned in the same or different plasmids.

In addition to the transcription construct, depending upon the manner of introduction of the transcription construct into the plant, other DNA sequences may be required. For example, when using the Ti- or Ri-plasmid for transformation of plant cells, as described below, at least the right border and frequently both the right and left borders of the T-DNA of the Ti- or Ri-plasmids will be joined as flanking regions to the transcription construct. The use of T-DNA for transformation of plant cells has received extensive study and is amply described in EPA Serial No. 120,516, Hoekema, In: The Binary Plant Vector System Offset-drukkerij Kanthers B.V., Alblasserdam, 1985, Chapter V, Knauf et al., Genetic Analysis of Host Range Expression by Agrobacterium, In: Molecular Genetics of the Bacteria-Plant Interaction, Puhler, A. ed., Springer-Verlag, NY, 1983, p.245, and An et al., EMBO J. (1985) 4:277-284

Alternatively, to enhance integration into the plant genome, terminal repeats of transposons may be used as borders in conjunction with a transposase. In this situation, expression of the transposase should be inducible, so that once the transcription construct is integrated into the genome, it should be relatively stably integrated and avoid hopping.

The transcription construct will normally be joined to a marker for selection in plant cells. Conveniently, the marker may be resistance to a biocide, particularly an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, chloramphenicol, or the like. The particular marker employed will be one which will allow for selection of transformed cells as compared to cells lacking the DNA which has been introduced.

A variety of techniques are available for the introduction of DNA into a plant cell host. These techniques include transformation with Ti-DNA employing A. tumefaciens or A. rhizogenes as the transforming agent, protoplast fusion, injection, electroporation,

etc. For transformation with Agrobacterium, plasmids can be prepared in E. coli which plasmids contain DNA homologous with the Ti-plasmid, particularly T-DNA. The plasmid may or may not be capable of replication in Agrobacterium, that is, it may or may not have a broad spectrum prokaryotic replication system, e.g., RK290, depending in part upon whether the transcription construct is to be integrated into the Ti-plasmid or be retained on an independent plasmid. By means of a helper plasmid, the transcription construct may be transferred to the A. tumefaciens and the resulting transformed organism used for transforming plant cells.

Conveniently, explants may be cultivated with the A. tumefaciens or A. rhizogenes to allow for transfer of the transcription construct to the plant cells, the plant cells dispersed in an appropriate selective medium for selection, grown to callus, shoots grown and plantlets regenerated from the callus by growing in rooting medium. The Agrobacterium host will contain a plasmid having the vir genes necessary for transfer of the T-DNA to the plant cells and may or may not have T-DNA. For injection and electroporation, disarmed Ti-plasmids (lacking the tumor genes, particularly the T-DNA region) may be introduced into the plant cell.

As a host cell, any of a number of fruit bearing plants may be employed in which the plant parts of interest are derived from the ovary wall. These include true berries such as tomato, grape, blueberry, cranberry, currant, and eggplant; stone fruits (drupes) such as cherry, plum, apricot, peach, nectarine and avocado; compound fruits (druplets) such as raspberry and blackberry. In hesperidium (oranges, citrus), the expression cassette might be expected to be expressed in the "juicy" portion of the fruit. In pepos (such as watermelon, cantelope, honeydew, cucumber and squash) the equivalent tissue for expression is most likely the inner edible portions, whereas in legumes (such as

peas, green beans, soybeans) the equivalent tissue is the seed pod.

Identifying useful transcriptional initiation regions may be achieved in a number of ways. Where a fruit protein has been or is isolated, it may be partially sequenced, so that a probe may be designed for identifying messenger RNA specific for fruit. To further enhance the concentration of the messenger RNA specifically associated with fruit, cDNA may be prepared and the cDNA subtracted with messenger RNA or cDNA from non-fruit associated cells. The residual cDNA may then be used for probing the genome for complementary sequences, using an appropriate library prepared from plant cells. Sequences which hybridize to the cDNA may then be isolated, manipulated, and the 5'-untranslated region associated with the coding region isolated and used in expression constructs to identify the transcriptional activity of the 5'-untranslated region. In some instances, a probe may be employed directly for screening a genomic library and identifying sequences which hybridize to the probe. The sequences will be manipulated as described above to identify the 5'-untranslated region.

As an example, a promoter of particular interest for the subject invention, the fruit-specific transcriptional initiation region (promoter) from a DNA sequence which encodes a protein described as 2A11 in the Experimental section was identified as follows. cDNA clones made from ripe fruit were screened using cDNA probes made from ripe fruit, green fruit, and leaf mRNA. Clones were selected having more intense hybridization with the fruit DNAs as contrasted with the leaf cDNAs. The screening was repeated to identify a particular cDNA referred to as 2A11. The 2A11 cDNA was then used for screening RNA from root, stem, leaf, and seven stages of fruit development after the mRNA was sized on gels. The screening demonstrated that the

particular message was present throughout the seven stages of fruit development. The mRNA complementary to the specific cDNA was absent in other tissues which were tested. The cDNA was then used for screening a genomic library and a fragment selected which hybridized to the subject cDNA. The 5' and 3' non-coding regions were isolated and manipulated for insertion of a foreign sequence to be transcribed under the regulation of the 2A11 promoter.

10 The cells which have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick et al., Plant Cell Reports (1986) 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or
15 different strains, identifying the resulting hybrid having the desired phenotypic characteristic. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested for use to provide
20 fruits with the new phenotypic property.

A protein is provided having the sequence described in the Experimental section designated as 2A11. This protein could be a storage protein and be useful in enhancing sulfur containing amino acids (cysteine
25 and methionine) in the diet. It can be obtained in substantially pure form by providing for expression in prokaryotes or eukaryotes, e.g., yeast by inserting the open reading frame into an expression cassette containing a transcriptional initiation region. A variety of
30 expression cassettes are commercially available or have been described in the literature. See, for example, U.S. Patent Nos. 4,532,207; 4,546,082; 4,551,433; and 4,559,302. The product, if intracellular, may be isolated by lysing of the cells and purification of the
35 protein using electrophoresis, affinity chromatography, HPLC extraction, or the like. The product may be isolated in substantially pure form free of other plant

products, generally having at least about 95% purity, usually at least about 99% purity.

The following examples are offered by way of illustration and not by limitation.

5

EXPERIMENTAL

Example 1

Construction of Tomato Ripe Fruit cDNA Bank and Screening for Fruit-Specific Clones

Tomato plants (Lycopersicon esculentum cv UC82B) were grown under greenhouse conditions. Poly(A)⁺RNA was isolated as described by Mansson et al., Mol. Gen. Genet. (1985) 200:356-361. The synthesis of cDNA from poly(A)⁺ RNA prepared from ripe fruit, cloning into the PstI site of the plasmid pUC9 and transformation into an E. coli vector were all as described in Mansson et al., Mol. Gen. Genet. (1985) 200:356-361.

Library Screening

Two thousand recombinant clones were screened by colony hybridization with radiolabeled cDNA made from tomato red fruit mRNA, immature green fruit mRNA, and leaf mRNA. Bacterial colonies immobilized onto GeneScreen Plus filters (New England Nuclear), were denatured in 1.5 M NaCl in 0.5 M NaOH, then neutralized in 1.5 M NaCl in 0.5 M Tris-HCl pH 8, and allowed to air dry. Hybridization, washing and autoradiography were all performed as described in Maniatis et al., Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor, New York.

Sixty-five clones were selected which had more intense hybridization signals with fruit cDNA than with leaf cDNA and therefore appeared to be under-represented in the leaf mRNA population relative to the fruit population. Replicate slot blot filters were prepared using purified DNA from the selected clones and hybrid-

ized with radioactive cDNA from leaf, green fruit, and red fruit as before. This allowed selection of cDNA clone 2A11, also referred to as pCGN1299 which is on at high levels in both the fruit stages (red and green) and off in the leaf.

Example 2

Analysis of Clones

Synthesis of RNA Probes

10: The cDNA insert of pCGN1299 was excised as an EcoRI to HindIII fragment of approximately 600 bp (as measured on an agarose gel), and subcloned into the Riboprobe vector pGEM1 (Promega Biotec), creating pCGN488. ³²P-labeled transcripts made from each strand
15 of the pCGN488 insert using either SP6 or T7 polymerase were used as probes in separate Northern blots containing mRNA from leaf, immature green and mature red fruits. The RNA transcript from the SP6 promoter did not hybridize to the tomato mRNA. However, the tran-
20 script from the T7 promoter hybridized to an mRNA of approximately 700 nt in length from the green fruit and the red fruit but not to mRNA from tomato leaf. The direction of transcription of the corresponding mRNA was thus determined.

25 The tissue specificity of the pCGN1299 cDNA was demonstrated as follows. RNA from root, stem, leaf, and seven stages of fruit development (immature green, mature green, breaker, turning, pink, light red, and red) was sized on formaldehyde/agarose gels accord-
30 ing to the method described by Maniatis et al., (1982), immobilized on nitrocellulose and hybridized to ³²P-labeled RNA which was synthesized in vitro from pCGN488 using T7 polymerase. Each lane contained 100 ng of polyA⁺ RNA except for two lanes (pink and light red
35 lanes) which contained 10 µg of total RNA. The Northern analysis of mRNA from root, stem, leaf, and various stages of fruit development indicated that

pCGN1299 cDNA was expressed in all stages of fruit development from the early stages immediately after anthesis to red ripe fruit. No mRNA hybridizing to pCGN1299 was found in leaf, stem, or root tissue. The size of the mRNA species hybridizing to the pCGN488 probe was approximately 700 nt.

Message abundance corresponding to the pCGN1299 cDNA was determined by comparing the hybridization intensity of a known amount of RNA synthesized in vitro from pCGN488 using SP6 polymerase to mRNA from red tomato fruit in a Northern blot. The ³²P-labeled transcript from pCGN488 synthesized in vitro using T7 polymerase was used as a probe. The Northern analysis was compared to standards which indicated that the pCGN1299 cDNA represents an abundant mRNA class in tomato fruit, being approximately 1% of the message.

Example 3

Sequencing of pCGN1299 and pCGN1298 cDNA Clones

DNA Sequencing

The polyA+ sequence was missing from pCGN1299 cDNA. A longer cDNA clone, pCGN1298, therefore was identified by its hybridization with the pCGN488 probe. The complete DNA sequence of the two cDNA inserts was determined using both Maxam-Gilbert and the Sanger di-deoxy techniques and is as follows. The sequence of pCGN1298 contains additional sequences at both the 5' and 3' end compared to pCGN1299. As shown in Figure 1, the sequences are identical over the region that the two clones have in common.

Amino Acid Sequence

The pCGN1299 cDNA sequence was translated in three frames. The longest open reading frame (which starts from the first ATG) is indicated. Both pCGN1299 and pCGN1298 have an open reading frame which encodes a

96 amino acid polypeptide (see Figure 1). The protein has a hydrophobic N-terminus which may indicate a leader peptide for protein targeting. A hydrophobicity profile was calculated using the Hopp and Woods, (Proc. Natl. Acad. Sci. USA (1981) 78:3824-3828) algorithm. Residues 10-23 have an extremely hydrophobic region. A comparison of 2A11 to pea storage proteins and other abundant storage proteins is shown in Figure 2. The sulfur-rich composite of the fruit-specific protein is similar to a pea storage protein which has recently been described (see Higgins et al., J. Biol. Chem. (1986) 261:11124-11130, for references to the individual peptides). This may indicate a storage role for this fruit-specific protein abundant species.

Example 4

Screening Genomic Library for Genomic Clones

Southern Hybridization

Southern analysis was performed as described by Maniatis et al., 1982. Total tomato DNA from cultivar UC82B was digested with EcoRI or HindIII, separated by agarose gel electrophoresis and transferred to nitrocellulose. Southern hybridization was performed using a ^{32}P -labeled probe produced by nick translation of pCGN488 (Maniatis et al., 1982). The simple hybridization pattern indicated that the gene encoding pCGN1299 cDNA was present in a few or perhaps even one copy in a tomato genome.

Isolation of a Genomic Clone

A genomic library established in Charon35/Sau3A constructed from DNA of the tomato cultivar VFNT-Cherry was screened using the [^{32}P]-RNA from cDNA clone pCGN488 as a probe. A genomic clone containing approximately 12.5 kb of sequence from the tomato genome was isolated. The region which hybridizes to a pCGN488

probe spans an XbaI restriction site which was found in the cDNA sequence and includes the transcriptional initiation region designated 2A11.

5 Sequence of Genomic Clone

 The DNA sequence of the genomic clone was determined by Sanger dideoxy techniques and is as shown in Figure 4. The sequence of the genomic clone is identical to the pCGN1299 cDNA clone over the region
10 they have in common.

Subcloning

 The region surrounding the XbaI restriction site, approximately 2.4 kb in the 5' direction and approximately 2.1 kb in the 3' direction was subcloned to
15 provide an expression cassette. The 5' XhoI to XbaI fragment and the 3' XbaI to EcoRI fragment from the 2A11 genomic clone were inserted into a pUC-derived chloromphenicol plasmid containing a unique XhoI site
20 and no XbaI site. This promoter cassette plasmid is called pCGN1273.

Example 5

25 Construction of Fruit-Specific Antisense Cassette

Insertion of Antisense Fragment

 The 2A11 genomic fragment was tagged with PG antisense sequences by insertion of PG into the unique XbaI site of the pCGN1273 promoter cassette in the anti-
30 sense orientation. The inserted sequences increased the size of the mRNA over the endogenous transcript, and thus the expression pattern of the construct could be compared to the endogenous gene by a single Northern hybridization in a manner analogous to the detection of
35 a tuber-specific potato gene described by Eckes et al., Mol. Gen. Genet. 1986 205:14-22.

Example 6

Insertion of Tagged Genomic Construction
Into Agrobacterium Binary Vectors

The tagged genomic construction is excised
5 using the flanking XhoI restriction enzyme sites and is
cloned into the unique SalI site of the binary plasmid
pCGN783 containing a plant kanamycin resistance marker
between the left and right borders to provide plasmid
pCGN1269.

10. This plasmid binary vector in E. coli C2110 is
conjugated into A. tumefaciens containing a disarmed
Ti-plasmid capable of transferring the polygalacturo-
nase antisense cassette and the kanamycin resistance
cassette into the plant host genome.

15 The Agrobacterium system which is employed is
A. tumefaciens PC2760 (G. Ooms et al., Plasmid (1982)
7:15-29; Hoekema et al., Nature (1983) 303:179-181;
European Patent Application 84-200239.6, 2424183).

20 1. Construction of pCGN783

pCGN783 is a binary plasmid containing the
left and right T-DNA borders of A. tumefaciens octopine
Ti-plasmid pTiA6 (Currier and Nester, J. Bacteriol.
(1976) 126:157-165) the gentamicin resistance gene of
25 pPH1J1 (Hirsch et al., Plasmid (1984) 12:139-141), the
35S promoter of cauliflower mosaic virus (CaMV)
(Gardner et al., Nucleic Acid Res. (1981) 9:1871-1880);
the kanamycin resistance gene of Tn5 (Jorgensen, Mol.
Gen. (1979) 177:65); and the 3' region from transcript
30 7 of pTiA6 (Currier and Nester, supra (1976)). A sche-
matic diagram of the construction of pCGN783 is shown
in Figure 3. (a) through (f) refer to the plasmid
constructions detailed below.

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(a) Construction of pCGN587

The HindIII-SmaI fragment of Tn5 containing the entire structural gene for APH3'II (Jorgensen et al., Mol. Gen. (1979) 177:65), was cloned into pUC8 (Vieira and Messing, Gene (1982) 19:259), converting the fragment into a HindIII-EcoRI fragment, since there is an EcoRI site immediately adjacent to the SmaI site. The PstI-EcoRI fragment containing the 3' portion of the APH3'II gene was then combined with an EcoRI-BamHI-SalI-PstI linker into the EcoRI site of pUC7 (pCGN546W). Since this construct does not confer kanamycin resistance, kanamycin resistance was obtained by inserting the BglI-PstI fragment of the APH3'II gene into the BamHI-PstI site (pCGN546X). This procedure reassembles the APH3'II gene, so that EcoRI sites flank the gene. An ATG codon was upstream from and out of reading frame with the ATG initiation codon of APH3'II. The undesired ATG was avoided by inserting a Sau3A-PstI fragment from the 5' end of APH3'II, which fragment lacks the superfluous ATG, into the BamHI-PstI site of pCGN546W to provide plasmid pCGN550. The EcoRI fragment of pCGN550 containing the APH3'II gene was then cloned into the EcoRI site of pUC8-pUC13 (K. Buckley supra (1985)) to give pCGN551.

Each of the EcoRI fragments containing the APH3'II gene was then cloned into the unique EcoRI site of pCGN451, which contains an octopine synthase cassette for expression to provide pCGN548 (2ATG) and pCGN552 (1ATG). The plasmid pCGN451 having the ocs 5' and the ocs 3' in the proper orientation was digested with EcoRI and the EcoRI fragment from pCGN551 containing the intact kanamycin resistance gene inserted with EcoRI site to provide pCGN552 having the kanamycin resistance gene in the proper orientation. This ocs/KAN gene was used to provide a selectable marker for the trans type binary vector pCGN587.

The 5' portion of the engineered octopine synthase promoter cassette consists of pTiA6 DNA from the XhoI at bp 15208-13644 (Barker et al., supra (1983)), which also contains the T-DNA boundary sequence (border) implicated in T-DNA transfer. In the plasmid pCGN587, the osc/KAN gene from pCGN552 provides a selectable marker as well as the right border. The left boundary region was first cloned in M13mp9 as a HindIII-SmaI piece (pCGN502) (base pairs 602-2212) and recloned as a KpnI-EcoRI fragment in pCGN565 to provide pCGN580. pCGN565 is a cloning vector based on pUC8-Cm, but containing pUC18 linkers. pCGN580 was linearized with BamHI and used to replace the smaller BglI fragment of pVCK102 (Knauf and Nester, Plasmid (1982) 8:45), creating pCGN585. By replacing the smaller SalI fragment of pCGN585 with the XhoI fragment from pCGN552 containing the ocs/KAN gene, pCGN587 was obtained.

(b) Construction of pCGN739 (Binary Vector)

To obtain the gentamicin resistance marker, the resistance gene was isolated from a 3.1 kb EcoRI-PstI fragment of pPHIJI (Hirsch et al., Plasmid (1984) 12:139-141) and cloned into pUC9 (Vieira et al., Gene (1982) 19:259-268) yielding pCGN549.

The pCGN549 HindIII-BamHI fragment containing the gentamicin resistance gene replaced the HindIII-BglII fragment of pCGN587 (for construction, see 6.1(a), supra) creating pCGN594.

The pCGN594 HindIII-BamHI region which contains an ocs-kanamycin-ocs fragment was replaced with the HindIII-BamHI polylinker region from pUC18 (Yanisch-Perron, Gene (1985) 33:103-119) to make pCGN739.

(c) Construction of 726c (1 ATG-Kanamycin-3' region)

pCGN566 contains the EcoRI-HindIII linker of pUC18 (Yanisch-Perron, ibid) inserted into the EcoRI-HindIII sites of pUC13-Cm (K. Buckley, Ph.D. Thesis,

University of California, San Diego, 1985). The HindIII-BglII fragment of pNW31c-8, 29-1 (Thomashow et al., Cell (1980) 19:729) containing ORF1 and 2 (Barker et al., Plant Mol. Biol. (1984) 2:335-350) was subcloned into the HindIII-BamHI sites of pCGN566 producing pCGN703.

The Sau3A fragment of pCGN703 containing the 3' region of transcript 7 from pTiA6 (corresponding to bases 2396-2920 of pTi15955 (Barker et al., supra (1984))) was subcloned into the BamHI site of pUC18 (Yanisch-Perron et al., supra (1985)) producing pCGN709.

The EcoRI-SmaI polylinker region of pCGN709 was replaced with the EcoRI-SmaI fragment from pCGN587 (see 6.1(a), supra) which contains the kanamycin resistance gene (APH3'II) producing pCGN726.

The EcoRI-SalI fragment of pCGN726 plus the BglII-SalI sites of pUC8-pUC13-cm (chloramphenicol resistant, K. Buckley, Ph.D. Thesis, University of California, San Diego, 1985) producing pCGN738. To construct pCGN734, the HindIII-SphI site of M13mp19 (Norrand et al., Gene (1983) 26:101-106). Using an oligonucleotide corresponding to bases 3287 to 3300, DNA synthesis was primed from this template. Following S1 nuclease treatment and HindIII digestion, the resulting fragment was cloned into the HindIII-SmaI site of pUC19 (Yanisch-Perron et al., supra (1985)). The resulting EcoRI to HindIII fragment of pTiA6 (corresponding to bases 3390-4494) into the EcoRI site of pUC8 (Vieira and Messing, supra (1982)) resulting in pCGN734. pCGN726c is derived from pCGN738 by deleting the 900 bp EcoRI-EcoRI fragment.

(d) Construction of pCGN167

pCGN167 is a construct containing a full length CaMV promoter, 1 ATG-kanamycin gene, 3' end and the bacterial Tn903-type kanamycin gene. MI is an EcoRI fragment from pCGN550 (see construction of pCGN587) and was cloned into the EcoRI cloning site in the 1 ATG-

kanamycin gene proximal to the polylinker region of M13mp9. See copending Application Serial No. 920,579, filed October 17, 1986, which disclosure is incorporated herein by reference.

5 To construct pCGN167, the AluI fragment of CaMV (bp 7144-7735) (Gardner et al., Nucl. Acids Res. (1981) 9:2871-2888) was obtained by digestion with AluI and cloned into the HincII site of M13mp7 (Vieira, Gene (1982) 19:259) to create C614. An EcoRI digest of C614
10 produced the EcoRI fragment from C614 containing the 35S promoter which was cloned into the EcoRI site of pUC8 (Vieira et al., Gene (1982) 19:259) to produce pCGN146. To trim the promoter region, the BglII site (bp 7670) was treated with BglII and Bal31 and subse-
15 quently a BglII linker was attached to the Bal31 treated DNA to produce pCGN147.

pCGN148a containing the promoter region, selectable marker (KAN with 2 ATGs) and 3' region was prepared by digesting pCGN528 (see below) with BglII
20 and inserting the BamHI-BglII promoter fragment from pCGN147. This fragment was cloned into the BglII site of pCGN528 so that the BglII site was proximal to the kanamycin gene of pCGN528.

The shuttle vector used for this construct, pCGN528, was made as follows. pCGN525 was made by
25 digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et al., Mol. Gen. (1979) 177:65) with HindIII-BamHI and inserting the HindIII-BamHI fragment containing the kanamycin gene into the
30 HindIII-BamHI sites in the tetracycline gene of pACYC184 (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156). pCGN526 was made by inserting the BamHI fragment 19 of pTiA6 (Thomashow et al., Cell (1980) 19:729-739) into the BamHI site of pCGN525. pCGN528
35 was obtained by deleting the small XhoI fragment from pCGN526 by digesting with XhoI and religating.

pCGN149a was made by cloning the BamHI kana-
mycin gene fragment from pMB9KanXXI into the BamHI site
of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and
Messing, Gene (1982) 19:259-268) which has the XhoI site
5 missing but contains a functional kanamycin gene from
Tn903 to allow for efficient selection in Agrobacterium.

pCGN149a was digested with BglII and SphI. .
This small BglII-SphI fragment of pCGN149a was replaced
with the BamHI-SphI fragment from MI (see below) iso-
10 lated by digestion with BamHI and SphI. This produces
pCGN167.

(e) Construction of pCGN766c (35S promoter-3' region)

The HindIII-BamHI fragment of pCGN167 contain-
15 ing the CaMV-35S promoter, 1 ATG-kanamycin gene and the
BamHI fragment 19 of pTiA6 was cloned into the BamHI-
HindIII sites of pUC19 (Norranders et al., supra (1985);
Yanisch-Perron et al., supra (1985)) creating pCGN976.

The 35S promoter and 3' region from transcript
20 7 was developed by inserting a 0.7 kb HindIII-EcoRI
fragment of pCGN976 (35S promoter) and the 0.5 kb
EcoRI-SalI fragment of pCGN709 (transcript 7:3' for
construction see supra) into the HindIII-SalI sites of
pCGN566 creating pCGN766c.

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(f) Final Construction of pCGN783

The 0.7 kb HindIII-EcoRI fragment of pCGN766c
(CaMV-35S promoter) was ligated to the 1.5 kb EcoRI-
SalI fragment of pCGN726c (1-ATG-KAN-3' region) into
30 the HindIII-SalI sites of pUC119 (J. Vieira, Rutgers
University, New Jersey) to produce pCGN778. The 2.2 kb
region of pCGN778, HindIII-SalI fragment containing the
CaMV 35S promoter (1-ATG-KAN-3' region) replaced the
HindIII-SalI polylinker region of pCGN739 to produce
35 pCGN783.

Example 7Transfer of Genomic Construction
to Tomato via Cocultivation

Substantially sterile tomato cotyledon tissue
5 is obtained from seedlings which have been grown at 24°C,
with a 16hr/8hr day/night cycle in 100x25 mm petri dishes
containing Murashige-Skoog salt medium and 0.8% agar
(pH 6.0). Any tomato species may be used, however,
here the inbred breeding line was UC82B, available from
10 the Department of Vegetable Crops, University of Cali-
fornia, Davis, CA 95616. The cotyledons are cut into
three sections and the middle placed onto feeder plates
for a 24-hour preincubation. The feeder plates are pre-
pared by pipetting 0.5 ml of a tobacco suspension cul-
15 ture (10^6 cells/ml) onto 0.8% agar medium, containing
Murashige minimal organic medium (K.C. Biologicals),
2,4-D (0.1 mg/l), kinetin (1 mg/l), thiamine (0.9 mg/l)
and potassium acid phosphate (200 mg/l, pH 5.5). The
feeder plates are prepared two days prior to use. A
20 sterile 3 mm filter paper disk containing feeder medium
is placed on top of the tobacco cells after the suspen-
sion cells are grown for two days.

Following the preincubation period, the middle
one third of the cotyledon sections are placed into a
25 liquid MG/L broth culture (1-5 ml) of the A. tumefaci-
ens strain. The binary plasmid pCGN1269 is transferred
to A. tumefaciens strain 2760 by conjugation or by
transformation selecting for Gentamicin resistance en-
coded by the plasmid pCGN1269. The cotyledon sections
30 are cocultivated with the bacteria for 48 hrs on the
feeder plates and then transferred to regeneration
medium containing 500 mg/l carbenicillin and 100 mg/l
kanamycin. The regeneration medium is a K.C. Biologi-
cals Murashige-Skoog salts medium with zeatin (2 mg/l)
35 myo-inositol (100 mg/l), sucrose (20 g/l), Nitsch vita-
mins and containing 0.8% agar (pH 6.0). In 2-3 weeks,
shoots are observed to develop. When the shoots are

approximately 1.25 cm, they are excised and transferred to a Murashige and Skoog medium containing carbenicillin (500 mg/l) and kanamycin (50 mg/l) for rooting. Roots develop within 10-12 days.

- 5 Shoots which develop and subsequently root on media containing the kanamycin are tested for APH3'II enzyme.

An aminoglycoside phosphotransferase enzyme (APH3'II) assay is conducted on putative transformed tomato plants and shoots. APH3'II confers resistance to kanamycin and neomycin. APH3'II activity is assayed (Reiss *et al.*, *Gene* (1984) 30:211-218) employing electrophoretic separation of the enzyme from other interfering proteins and detection of its enzymatic activity by *in situ* phosphorylation of kanamycin. Both kanamycin and [γ -³²P] ATP act as substrates and are embedded in an agarose gel which is placed on top of the polyacrylamide gel containing the proteins. After the enzymatic reaction, the phosphorylated kanamycin is transferred to P-81 phosphocellulose ion exchange paper and the radiolabeled kanamycin is finally visualized by autoradiography. The Reiss *et al.* method is modified in the final washing of the P-81 ion exchange paper by rinsing in 0.1 mg/ml of proteinase K.

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Example 8

Construction of Tagged 2A11 Plasmids

In Binary Vectors

The complete sequence of the 2A11 genomic DNA cloned into pCGN1273 from the XhoI site (position 1 at the 5' end) to the EcoRI site (position 4654) is shown in Figure 4.

pCGN1267 was constructed by deleting from pCGN1273 a portion of the plasmid polylinker from the EcoRV site to the BamHI site. Two DNA sequences were inserted into pCGN1273 at the unique XbaI site (position 2494). This site is in the 3' non-coding region of the 2A11 genomic clone before the poly A site.

PCGN1273 was tagged with 360 bp (from base number 1 to 360) from the 5' region of the tomato polygalacturonase (PG) cDNA clone, Fl (Sheehy *et al.*, *Mol. Gen. Genet.* (1987) 208:30-36) at the unique XbaI restriction enzyme site. The tag was inserted in the antisense orientation resulting in plasmid pCGN1271 and in the sense orientation yielding plasmid pCGN1270. Each plasmid was linearized at the unique BglII restriction enzyme site and cloned into the binary vector pCGN783 at the unique BamHI restriction enzyme site.

PCGN1273 was also tagged with a 0.5 kb fragment of DNA (base number 1626 to 2115) from a PG genomic clone (see Figure 5) which spans the 5' end of the intron/exon junction. This fragment was cloned into the XbaI site resulting in plasmid pCGN1215. pCGN1215 was linearized at the unique BglII site and cloned into pCGN783 at the BamHI site resulting in two plasmids, pCGN1219 and pCGN1220, which differ only in the orientation of pCGN1215 within pCGN783.

Three DNA sequences were inserted into pCGN1267 at the unique ClaI sites (position 2402, 2406). These sites are in the 3' non-coding region of the 2A11 genomic clone, 21 bp from the stop codon. The 383 bp XbaI fragment from the PG cDNA clone was cloned into the ClaI site of pCGN1267 after filling in the XbaI and ClaI ends with Klenow and blunt ligation. The fragment in a sense orientation resulted in plasmid pCGN1263 and in the antisense orientation gave pCGN1262. pCGN1263 was linearized at the unique BglII site and cloned into pCGN783 at the BamHI site yielding pCGN1260. pCGN1262 was also linearized at the BglII site and cloned into pCGN783 at the BamHI site resulting in two plasmids, pCGN1255 and pCGN1258, which differ only in the orientation of pCGN1262 in the binary vector pCGN783.

The 0.5 kb fragment of the PG genomic clone spanning the intron/exon junction (*supra*) was cloned into pCGN1267 at the ClaI site in an antisense direc-

tion yielding plasmid pCGN1225. This plasmid was linearized at the BglII restriction enzyme site and cloned into pCGN783 at the BamHI site producing two plasmids, pCGN1227 and pCGN1228, which differ only in the orientation of pCGN1225 in the binary vector.

The Eco7 fragment (base numbers 5545 to 12,823) (Barker et al., Plant Mol. Biol. (1983) 2:335-350) from the octopine plasmid pTiA6 of A. tumefaciens (Knauf and Nester, Plasmid (1982) 8:45-54) was subcloned into pUC19 at the EcoRI site resulting in plasmid pCGN71. A RsaI digest allowed a fragment of DNA from bases 8487 to 9036 of the Eco7 fragment to be subcloned into the vector m13 BlueScript Minus (Stratagene, Inc.) at the SmaI site resulting in plasmid pCGN1278. This fragment contains the coding region of the genetic locus designated tmr which encodes a dimethylallyl transferase (isopentenyl transferase) (Akiyoshi et al., Proc. Natl. Acad. Sci. USA (1984) 81:5994-5998; Barry et al., ibid (1984) 81:4776-4780). An exonuclease/mung bean treatment (Promega Biotech) produced a deletion on the 5' end of the tmr gene to a point 39 base pairs 5' of the start codon. The tmr gene from pCGN1272 was subcloned into the ClaI site of pCGN1267. The tmr gene in the sense orientation yielded pCGN1261 and in the antisense orientation gave plasmid pCGN1266. pCGN1261 was linearized at the BglII site and cloned into pCGN783 at the BamHI site resulting in plasmid pCGN1254. pCGN1266 was also linearized at the BglII site and subcloned into pCGN783 at the BamHI site yielding two plasmids, pCGN1264 and pCGN1265, which differ only in the orientation of pCGN1266 in pCGN783.

Analysis of Expression in Transgenic Plants

Immature green fruit (approximately 3.2 cm in length) was harvested from two tomato plants cv. UC82B that had been transformed with a disarmed Agrobacterium

strain containing pCGN1264. Transgenic plants are designated 1264-1 and 1264-11. The pericarp from two fruits of each plant was ground to a powder under liquid N₂, total RNA extracted and polyA⁺ mRNA isolated (as described in Mansson *et al.*, *Mol. Gen. Genet.* (1985) 200:356-361). Young green leaves were also harvested from each plant and polyA⁺ mRNA isolated.

Approximately 19 µg of total RNA from fruit, 70 ng of polyA⁺ mRNA from fruit and 70 ng of polyA⁺ mRNA from leaves from transformed plants 1264-1 and 1264-11 was run on a 0.7% agarose formaldehyde Northern gel and blotted onto nitrocellulose (Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual* (1982) Cold Spring Harbor, New York). Also included on the gel as a negative control was approximately 50 ng of polyA⁺ mRNA from leaf and immature green fruit of a nontransformed UC82B plant.

As a positive control and to help in quantitating mRNA levels, *in vitro* transcribed RNA from pCGN1272 was synthesized using T3 polymerase (Stratagene, Inc.). Nineteen pg and 1.9 pg of this *in vitro* synthesized RNA were loaded on the Northern gel.

The probe for the Northern filter was the 1.0 kb *tmr* insert DNA (a *Kpn*I to *Sac*I fragment) from pCGN1272 isolated by electroelution from an agarose gel (Maniatis, *supra* (1982)) and labeled by nick translation (Bethesda Research Laboratory kit) using α³²P dCTP (Amersham).

The Northern filter was prehybridized at 42°C for 5 hrs in the following solution: 25 ml formamide, 12.5 ml 20X SSC, 2.5 ml 1 M NaP, 5 ml 50X Denhardts, 0.5 ml 10% SDS, 1 ml 250 mM EDTA, 1 ml 10 mg/ml ssDNA and 2 ml H₂O. Then one-fifth volume of 50% dextran sulfate and approximately 2.2X 10⁷ cpm of the probe was added and hybridization was for 15 hrs at 42°C.

The Northern filter was washed one time in 2X SSC and 0.1% SDS at 55°C for 20 minutes each wash. The filter was allowed to air dry before being placed with Kodak XAR film and an intensifying screen at -70° for two days.

Northern Results on Transgenic Plants

The nicked tmr probe hybridized with a mRNA species approximately 1.7 kb in length was observed in the total RNA and polyA⁺ mRNA fruit lanes of the Northern blot. This is the expected length of the reintroduced 2A11 gene (0.7 kb) tagged with the tmr gene (1.0 kb) in the antisense orientation. The level of expression from the reintroduced tagged gene is somewhat lower than the level of expression of the endogenous 2A11 gene. The level of expression of the reintroduced gene in immature green fruit is higher than the expression level in leaf tissue with a small amount of hybridizing mRNA in leaf tissue in these transformants.

Example 9

Screening Genomic Library for Polygalacturonase Genomic Clones

Isolation of a Genomic Clone

An EcoRI partial genomic library established in Charon 4 constructed from DNA of a Lycopersicon esculentum cultivar was screened using a probe from the polygalacturonase cDNA (Sheehy et al., Mol. Gen. Genet. (1987) 208:30-36). A lambda clone containing an approximately 16 kb insert was isolated from the library, of which an internal 2207 bp HindIII to EcoRI was sequenced. The HindIII-EcoRI fragment includes the polygalacturonase promoter region.

Sequence of Genomic Clone

The DNA sequence of the genomic clone was determined by Sanger dideoxy techniques and is as shown in Figure 5. The sequence of the genomic clone bases 1427 to 1748 are homologous to the polygalacturonase cDNA sequence.

The above results demonstrate the ability to identify inducible regulatory sequences in a plant genome, isolate the sequences and manipulate them. In this way, the production of transcription cassettes and expression cassettes can be produced which allow for differentiated cell production of the desired product. Thus, the phenotype of a particular plant part may be modified, without requiring that the regulated product be produced in all tissues, which may result in various adverse effects on the growth, health, and production capabilities of the plant. Particularly, fruit-specific transcription initiation capability is provided for modifying the phenotypic properties of a variety of fruits to enhance properties of interest such as processing, organoleptic properties, storage, yield, or the like.

E. coli strain pCGN1299x7118 was deposited with the American Type Culture Collection (A.T.C.C.), 12301 Parklawn Drive, Rockville, Maryland, 20852 on May 21, 1987 and given Accession No. 67408.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A DNA construct comprising in the direction of transcription, a fruit-specific transcriptional initiation region from a gene expressed at or immediately after anthesis or at the breaker stage, said gene remaining expressed at least until the ripe period, joined to a DNA sequence of interest other than the wild-type sequence associated with said initiation region, wherein said DNA sequence of interest is under the transcriptional regulation of said initiation region, and a transcriptional termination region.
2. A DNA construct according to Claim 1, wherein said transcriptional initiation region is from a gene expressed immediately upon anthesis.
3. A DNA construct according to Claim 1, wherein said transcriptional initiation region regulates transcription of a gene encoding a plant storage protein.
4. A DNA construct according to Claim 3, wherein said transcriptional initiation region is the 2A11 region.
5. A DNA construct according to Claim 1, wherein said DNA sequence of interest is a sequence complementary to a native plant transcript.
6. A DNA construct according to Claim 1, wherein said DNA sequence of interest is an open reading frame encoding an amino acid sequence of interest.
7. A DNA construct according to Claim 1, wherein said DNA sequence of interest is a polygalacturonase gene or fragment thereof of at least 12nt in the anti-sense direction.

8. A DNA construct for integration into a plant genome comprising at least the right T-DNA border joined to a DNA construct according to Claim 1.

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9. A DNA construct comprising in the direction of transcription, the fruit-specific transcriptional initiation region of a plant storage protein being active at or immediately after anthesis and remaining active until at least until the ripe period, joined to a DNA sequence other than the wild-type sequence, wherein said sequence comprises a unique restriction site for insertion of a sequence of interest to be under the transcriptional regulation of said initiation region, and a transcriptional termination region.

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10. A DNA construct according to Claim 9, wherein said transcriptional initiation region is the 2A11 region.

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11. A DNA construct for integration into a plant genome comprising at least the right T-DNA border joined to a DNA construct according to Claim 10.

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12. A DNA vector comprising a broad spectrum prokaryotic replication system and a DNA construct according to Claim 1.

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13. A DNA vector comprising a broad spectrum prokaryotic replication system and a DNA construct according to Claim 9.

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14. A method for specifically modifying the phenotype of fruit substantially distinct from other plant tissue, said method comprising:

transforming a tomato plant cell with a DNA construct under genomic integration conditions, wherein said DNA construct comprises in the direction of transcription, a 2A11 fruit-specific transcriptional initiation region, joined to a DNA polygalacturonase gene sequence, wherein said sequence is oriented in the antisense direction and under the transcriptional regulation of said initiation region, and a transcriptional termination region, whereby said DNA construct becomes integrated into the genome of said plant cell, whereby said antisense sequence is transcribed and inhibits expression of polygalacturonase in fruit;

regenerating a plant from said transformed plant cell; and
growing said plant to produce fruit of the modified phenotype.

15. A method according to Claim 14, wherein said transcription initiation region is the 2A11 region.

16. A plant cell comprising a DNA construct according to Claim 1.

17. A plant cell comprising a DNA construct according to Claim 9.

18. A method for specifically modifying the phenotype of tomato fruit substantially distinct from other plant tissue, said method comprising:

transforming a plant cell with a DNA construct under genomic integration conditions, wherein said DNA construct comprises in the direction of transcription, a fruit-specific transcriptional initiation region being active at or immediately after anthesis, said gene remaining active at least until the ripe period, joined to a DNA sequence other than the wild-type sequence and capable of modifying the phenotype of fruit cells upon

transcription, wherein said sequence is under the transcriptional regulation of said initiation region, and a transcriptional termination region, whereby said DNA construct becomes integrated into the genome of said plant cell;

regenerating a plant from said transformed plant cell; and

growing said plant to produce fruit of the modified phenotype.

19. A plant comprising a DNA construct according to Claim 1.

20. A plant comprising a DNA construct according to Claim 9.

21. Fruit comprising a construct according to Claim 1.

22. Fruit according to Claim 21, wherein said fruit is tomato.

23. Fruit according to Claim 22, wherein said DNA sequence of interest is a polygalacturonase gene or fragment of at least 12nt thereof oriented in the anti-sense direction and said transcription initiation region is 2A11.

24. Fruit according to Claim 21, wherein said transcription initiation region is 2A11.

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3H11 TTTTTTTGAGCAAAGGGCAACTCAGATATCCAAAGATGAATCCACATATAGCTTACAGCTGGGAGAAC 69

3H11 ATTGTCTAACTCTTCTGAAATTTAAATGTTATCCAGAATCCTTCATCATAAAATAATATCAAAATGCAA 138

3H11 ATCTATTTTTTCTACTCTTGTCTAGCTTCAAGCTTCTTCTTCTGCTCATCAATTAGCAATTAATCCAAA 207
2A11 TGCTCATCAATTAGCAATTAATCCAAA

3H11 ACCATTATGGCTGCCAAAAATTGAGAGATGAAGTTTGCTATCTTCTTCTGTTGTTCTTTTGACGACCACT 276
2A11 ACCATTATGGCTGCCAAAAATTGAGAGATGAAGTTTGCTATCTTCTTCTGTTGTTCTTTTGACGACCACT
METAlaAlaLysAsnSerGluMETLysPheAlaIlePhePheValValLeuLeuThrThrThr

3H11 TTAGTTGATATGCTGGAATTCGAAAATGCAAGTGATGGCTCTTCGAGACATACCCCCACAAGAAACA 345
2A11 TTAGTTGATATGCTGGAATTCGAAAATGCAAGTGATGGCTCTTCGAGACATACCCCCACAAGAAACA
LeuValAspMETSerGlyIleSerLysMETGlnValMETAlaLeuArgAspIleProProGlnGluThr

3H11 TTGCTGAAAATGAAGCTACTTCCCACAAATATTTTGGGACTTTGTAACGAACCTTGACGCTCAAACCT 414
2A11 TTGCTGAAAATGAAGCTACTTCCCACAAATATTTTGGGACTTTGTAACGAACCTTGACGCTCAAACCT
LeuLeuLysMETLysLeuLeuProThrAsnIleLeuGlyLeuCysAsnGluProCysSerSerAsnSer

3H11 GATTGCATCGGAATTACCCTTTGCCAATTTTGTAAGGAGAAGACGGACCACTATGGTTTAAACATACCGT 483
2A11 GATTGCATCGGAATTACCCTTTGCCAATTTTGTAAGGAGAAGACGGACCACTATGGTTTAAACATACCGT
AspCysIleGlyIleThrLeuCysGlnPheCysLysGluLysThrAspGlnTyrGlyLeuThrTyrArg

3H11 ACATGCAACCTGTTGCCTTGAACAATATCAATGATCTATCGATCGATCTATCTATCTATTATCTGTCT 552
2A11 ACATGCAACCTGTTGCCTTGAACAATATCAATGATCTATCGATCGATCTATCTATCTATTATCTGTCT
ThrCysAsnLeuLeuPro

3H11 CTGCCGCTATAGTGTGTCTGTACCTTTGGTGTGAAGAATATGAATAAAGGGATACATATCTAGATA 621
2A11 CTGCCGCTATAGTGTGTCTGTACCTTTGGTGTGAAGAATATGAATAAAGGGATACATATCTAGATA

3H11 TATTCTAGGTAATGTCCTATTGTATTAAAAATTTGTAGCAATGATTGTTTGAATAAAAAACATACCATGA 690
2A11 TATTCTAGGTAATGTCCTATTGTATTAAAAATTTGTAGCAATGATTGTTTGAATAAAAAACATACCATGA

3H11 GTGAAATAATTATCCACATTAATTCAGTATTTATTTCACTTATGATACGTATTTTGTTCCTTTCCG 759
2A11 GTGAAATAATTATCC

3H11 GTAAAAAAAAAAAAA 774

FIGURE 1

2/8

(a)

2A11	ⓂMALRDIPPQETLL
PA1b	ⓂC SPFDIPPCGSPLCRCI
Chick pea inhibitor	ⓂC T-KSIPP ----QCRCN
Lima bean inhibitor	L C T-KSIPP ----QCRCT
α_1 -antitrypsin	LGAIPMSIPPEV

↑

(b)

2A11	TNILGLCNEPCSSNSDCI
PA1b	GSPLCRCPAGLVIGNCR
Barley chloroform/ methanol-soluble protein d	TNLLGNCR-FYLVQQTCA
Wheat α -amylase inhibitor 0.28	VSALTGCR-AMVKLQ-CV
Wheat albumin	VPALPACRPL-LRLQ-CN
Millet bi-functional inhibitor	NNPLDSCRWYVS ^A _{ER} -TACG
Castor bean 2S small subunit	QQNLRQCQEYIKQQVSGQ
Napin small subunit	AQNLRACQQWLNKQAMQS

FIGURE 2

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2A11 GENOMIC

10	20	30	40	50	60	70
CTCGAGCCCT	TTAAAAAGTA	TAGTCAATAT	TTAGMCTGAC	CGTGAATTTC	TTAATTATGA	TATATAATTT
80	90	100	110	120	130	140
AAAAGAAATC	ATGATCACAT	TCTACTGATG	AGAACATGTC	CTAATCAAGN	GAAGACATCG	ATGTGAAAAA
150	160	170	180	190	200	210
TACTTTTGT	TAAAAGTAAA	AAAAAATGTG	AAATTTTGT	AGTTATTTAC	LLCCTATACA	TTATTGACT
220	230	240	250	260	270	280
ATGTGCAAAC	TTTACAAATA	CCTAATAGAA	CATTTTCACC	TGCCTGTATA	TATGTAAAT	AATTATAATC
290	300	310	320	330	340	350
AACACTCTCA	CATAAAATAA	TTATCAGTAT	ATACATTAAT	ACTTGCCCTC	CACAAATGAAT	TAAATAAAAT
360	370	380	390	400	410	420
GTAGAACATG	ATCTACACTT	CAATAAAACT	AAGACCATAA	AGAATAATTT	CAAAATATAC	ACATGTCAAC
430	440	450	460	470	480	490
AATAAATTAT	TTGCATATTA	TATTAACCTA	CTAAAGCAATC	TTTACTTTTG	AAATATAAAA	ATAATCAAGT
500	510	520	530	540	550	560
TATAAGTCTG	CTCAAAGTAA	AGNACTTGTT	AGACTCATCT	GATTTTGAGA	AGGTAAGCAA	ATTGATGGTG
570	580	590	600	610	620	630
CATTAATAGT	ACAAGTAAAA	TATAAATAG	ATTTCATTAG	TAAAATTGTT	TTTTACTTTT	TTTATATATA
640	650	660	670	680	690	700
ATTATCAATA	TCCTTCAATG	GTAGGTTAAT	TATATTGTTA	ACTTCTTGTT	GAATTAAAGC	AATAAGACAA
710	720	730	740	750	760	770
GAATATTAAA	GATAAAGAA	CAATAAAAT	AGAAAGACTA	AGAGATAAGA	GTTTTCTTAT	TCTTCTTTCA
780	790	800	810	820	830	840
ATAAGTATCA	TCAAGTGTAT	ACAATATAAA	TTTTTGTAAT	TTTGATCTAT	CTATTATATA	TGTTATATAT
850	860	870	880	890	900	910
AAGCATACAA	AAGATCAGTC	ATAAATATGA	CTTTAATCAT	GAAAATAATG	AAAGACATTA	TGAAGGCGTA
920	930	940	950	960	970	980
AGGTTACTAG	AATAATAGTC	ATTAAAAAAA	GGGCTTATCT	TTATAATTGA	ATAATTGATG	AAGTAATGGA
990	1000	1010	1020	1030	1040	1050
GATAATTAGT	GAGCATAAAT	TTTTTTAAAA	AAATGGACAT	TTACACTATA	ATATTTTATA	ACACTTTCCC
1060	1070	1080	1090	1100	1110	1120
TTAAACATCT	AGGTATAAAT	AATGAGTCTT	GTCAAATCT	TAGTAGGAAA	AATTCTGTGA	AATTTTTTTA
1130	1140	1150	1160	1170	1180	1190
GTGAAAACAA	ATGATATAAA	TATCTTGAAT	ACTCATTATT	TGTTGTCTCA	TTAAAAATCT	TATCTGACCT
1200	1210	1220	1230	1240	1250	1260
ATAAAATAAA	TTATTGCTC	AATCATAAAT	AGTTTTTCAT	TCTAAAAATTA	GTATAATTAT	TAGTGAATAT
1270	1280	1290	1300	1310	1320	1330
TTAATTAACA	TAATTGTATA	CTAAGGGGCC	TATAAATTGG	ATTCTTCTCA	AAGAAAAATA	AAATCACCAC
1340	1350	1360	1370			1393
ACAACITTTCT	TCTTCTGCTC	ATCAATTAGC	AATTAATCCA	AAACCATT	ATG GCT GCC AAA AAT	
					MET Ala Ala Lys Asn	

FIGURE 4

(Page 1 of 3)

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1408 1423 1438 1454
 TCA GAG ATG AAG TTT GCT ATC TTC TTC GTT GTT CTT TTG ACC ACC ACT TTA CGTTCACAAC
 Ser Glu MET Lys Phe Ala Ile Phe Phe Val Val Leu Leu Thr Thr Leu
 1464 1474 1484 1494 1504 1514 1524
 ACTTCTCCCT TATTTTGTTT TCTTAATTC TTGGAAGTCA TATGCAITGT TTGCTATCA TCGTATATAT
 1534 1544 1554 1564 1574 1584 1594
 ATAAAGGAAA ATATTTTTCI TAATTACTCG TTTCTAATG TTGCTACGT AATCGGAAAT TATTATGAGA
 1604 1614 1624 1634 1644 1654 1664
 TAATGAACIT GCAAAGTCAT TATTATATAA CTTTTTTTTT ATACTTTCAT TTAAGAATTC ATTTTCTCA
 1674 1684 1694 1704 1714 1724 1734
 TTTTATATAA ACTTATTTT CAACAGAAAA TATTTTTCGA ACTATTCAAA CACACCCTAA GACATTACAT
 1744 1754 1764 1774 1784 1794 1804
 ATATATATAT ATACACCCTC CGTTTATAT TACTTAAIGC CTATTGAGTT GGGCCACCTT TTAAGAATGA
 1814 1824 1834 1844 1854 1864 1874
 TTCAATTAGA GATATGTTT ACTAAATTAA CCTATGCTTT AAGACTCTAA ATTGCGCTAT TACTATTTTA
 1884 1894 1904 1914 1924 1934 1944
 CGTTGTAATT TAATGACAAA CATTTCATAA TGACTATAGT CTGAACITAA TTAGACAGAC GTATCTATAG
 1954 1964 1974 1984 1994 2004 2014
 TTGCTTACT AATGATTCAT AGCTATATAT TTGGAGAGGA GAGAGCAAAA CGATATTAAAG AAAGGGAGGA
 2024 2034 2044 2054 2064 2074 2084
 GAGAGCCGAG GTAAATCTGA AATAGAGAAG AGAAAGGCAA CCAATTTTGA TCATCTATCA TACTTTTGAT
 2094 2104 2114 2124 2134 2144 2154
 TATTATTTT ATTATATGA CGTTTACATT ACAGTTTTCG AATTCTTACA TTAATCTTAA TCATAATATA
 2173 2188 2203
 TACA GTT GAT ATG TCT CGA ATT TCG AAA ATG CAA GTG ATG GCT CTT CGA GAC ATA
 Val Asp MET Ser Gly Ile Ser Lys MET Gln Val MET Ala Leu Arg Asp Ile
 2218 2233 2248 2263
 CCC CCA CAA GAA ACA TTG CTG AAA ATG AAG CTA CTT CCC ACA AAT ATT TTG GCA
 Pro Pro Gln Glu Thr Leu Leu Lys MET Lys Leu Leu Pro Thr Asn Ile Leu Gly
 2278 2293 2308
 CTT TGT AAC GAA CCT TCC AGC TCA AAC TCT GAT TGC ATC CGA ATT ACC CTT TCC
 Leu Cys Asn Glu Pro Cys Ser Ser Asn Ser Asp Cys Ile Gly Ile Thr Leu Cys
 2323 2338 2353 2368
 CAA TTT TGT AAG GAG AAG ACG GAC CAG TAT GGT TTA ACA TAC CGT ACA TCC AAC
 Gln Phe Cys Lys Glu Lys Thr Asp Gln Tyr Gly Leu Thr Tyr Arg The Cys Asn
 2383 2393 2403 2413 2423 2433
 GTG TTG CCT TGA ACAATATCAA TCATCTATCG ATCGATCTAT CTATCTATTT ATCTGTCTCT
 Leu Leu Pro
 2443 2453 2463 2473 2483 2493 2503
 GCGCGTATAG TGTGTCTGT ACCTTTGGTG TGAAGAATAT GAATAAAGCG ATACATATAT CTAGATATAT
 2513 2523 2533 2543 2553 2563 2573
 TCTAGGTAAT GTCCTATTGT ATTAAAAATT TGTAGCAATG ATTGTTTGAA TAAAAACATA CATTGAGTGA
 2583 2593 2603 2613 2623 2633 2643
 AATAATTATT CCACATTAAAT TCACGTATTT ATTCACTTA TCATACGTAT TTTTGTTCCT TTCCCGTAGA
 2653 2663 2673 2683 2693 2703 2713
 TTTTGTATCC TTTTCCCTTT TGAATATTAA ACATTAAGCA CAAATAATGT TTATTAATTT AAGTTAATAT

FIGURE 4

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2723	2733	2743	2753	2763	2773	2783
TTTTATTAG	CTATTATAT	TTTTATTGA	AATCAAACTT	GATAAATATT	TATAAAGATA	ATTAACAAGT
2793	2803	2813	2823	2833	2843	2853
AATGTGACAC	TAACACCATG	TAATATTATC	TTGTGCTTAT	TTATGATAAT	ATTTTAAAAAT	TATAATTTC
2863	2873	2883	2893	2903	2913	2923
GTAAAAAAT	TATTAATAAA	ACATACITTT	AAAAAGTGAG	TTAGCGTCCG	CTACCCACAT	ACTTATGAAT
2933	2943	2953	2963	2973	2983	2993
TGGACTAGTT	GTITTTTGAC	CCACAAAAAG	AATCGGCTAA	TTAAACCTGA	CCTATCAAAAT	TTGAGATCT
3003	3013	3023	3033	3043	3053	3063
GCATAGATTA	GTCCGAACGA	AATGAGTCAG	CCCGTATTGA	ACAAAATATC	AACAAGGACG	TTATGTAAAG
3073	3083	3093	3103	3113	3123	3133
ATGTTTAAGA	AGGAAAAAAG	ATTTCTAATA	CATATGGACT	TTCAATATCC	CAACTTTGTC	TGGCGATCTG
3143	3153	3163	3173	3183	3193	3203
AACCCTGCTT	AGTTTGTTGA	TCATTAACCT	GTCTTGCTAT	GTATTTAAGA	TTTAAACTTT	ATATGTTTAA
3213	3223	3233	3243	3253	3263	3273
AGTTACAGAA	AATACATATA	AATCTCTCAA	GACTTGCGAA	CATAATTAC	TTTAGTACTT	AAACTACATG
3283	3293	3303	3313	3323	3333	3343
AAAAATTTAA	TATCCTTTTA	ACATCTTTGA	AGTGAATTA	ATTATCACAA	TCCGAGCCTA	CACCTTGGAC
3353	3363	3373	3383	3393	3403	3413
GTGCGCGGCA	CTCAAGAACC	AGTCTGCTGC	CCCAAGCTAA	CCCTCATCCT	GACTGACTAC	AAGCGGAAGG
3423	3433	3443	3453	3463	3473	3483
CTAACTTAAG	TATACAAAAG	CTTAAACTG	AATAAAATA	ACTTTACAAG	GTTTTAACAG	AAATGAACAA
3493	3503	3513	3523	3533	3543	3553
CTTTGAAGAA	AATAATATAT	TCAACTAGCC	ATAAAAATA	CAACTTTAGT	CITTAATAAA	TTTAATAAAA
3563	3573	3583	3593	3603	3613	3623
TAAATGCAAA	ATATAGACTC	CITTAACTAA	CTGACTATCT	ATGAGCCCTC	TAAATGATAA	AGATGGAAGT
3633	3643	3653	3663	3673	3683	3693
CGGACACAAG	CCACGACATC	CTGACTAAAC	TCAGAAGTAA	ATAAAAATCC	CCGCAAAAAA	AGGAGCCCTA
3703	3713	3723	3733	3743	3753	3763
CCATGCGCTAA	CTCGAAGCTG	GGGATATATC	AATGAAGCTC	CTGTTGATGA	TCTTGAGAC	ATGTCTCTGC
3773	3783	3793	3803	3813	3823	3833
ATCATCAAAA	AGATGCAAGC	CAATGCTC	AGTACCTAAA	ATGTACGAGT	ATGTAAGGGA	AATCTATAAG
3843	3853	3863	3873	3883	3893	3903
TATAACATAA	GCTTGATACT	TCAATAAAAG	CAACATAC	TACCTCTTTT	CAACTCAACT	CAATTAAGA
3913	3923	3933	3943	3953	3963	3973
ATAAGTACT	CAACTCAAAG	ATTAGGTATT	CAACGCAAA	ATGGCACTCT	ACTCAATCAA	GTACAAATTA
3983	3993	4003	4013	4023	4033	4043
ACTCAGGATA	CTCGACTTAA	GATAGTCAAC	TCCGACACT	CAACTGAAGT	CATTTCAATA	TAAAGCAAGT
4053	4063	4073	4083	4093	4103	4113
TAAACAAAGT	TCAGTATAAA	GTAAAGTTGT	TTAAAAACAT	GATGTCAACT	CTGTGTGTAT	AATAAGGGAT
4123	4133	4143	4153	4163	4173	4183
ACAACATAAC	TTTGAAATGT	ATATAAAAA	ACAATTAAGT	GATGTATATA	AAAAACATT	AATCTATCCG
4193	4203	4213	4223	4233	4243	4253
AGATTCTCTA	ACCGACAACC	ATCACTTAAG	GGCTAAGATG	ATGATATAGC	GATCTACCGC	ACCGTCCCAT
4263	4273	4283	4293	4303	4313	4323
CCCATCTTAT	ACCGGGCCAA	AGGTATAAGA	CCTGAAGTCC	CTAATGAATC	CACATAATAA	CTGTTAAAG
4333	4343	4353	4363	4373	4383	4393
GAATCATCTA	AAAAGTATGA	CCCTTTTCTA	CCCATAGTGG	CTAACATGGT	TTATGGGGGG	TGTGAGTTAT
4403	4413	4423	4433	4443	4453	4463
CTGAAGTCTC	CCCCATATCG	GTGCTCAATA	CTACTCCAAA	AAATATACTG	CTCTTATGTT	TAAAAACATA
4473	4483	4493	4503	4513	4523	4533
CTGATTCTGT	GCTTTGAAAT	TATTGCTTAA	AGCTTAGATT	TTTGAAAAGC	TCCTTTTGA	AAATCGTAGT
4543	4553	4563	4573	4583	4593	4603
TTCTTTTTC	TTCTATTAA	GCTAGACATA	GGCTATGTAG	AACCTACCTT	TACCTTCCTT	CTCAAAAGTT
4613	4623	4633	4643	4653		
TGAAAACATT	TGCTTAGATT	CTTAGGGACT	ACTTAGTTCC	CTTGTGGAA	TTC	

FIGURE 4

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10	20	30	40	50	60	70
AAGCTTCTTA	AAAAGGCAAA	TGGATTAAAT	TGAAGTCAAA	ATAATTAAAT	ATAACAGTGG	TAAAGCAGCT
80	90	100	110	120	130	140
TAAGAAACCA	TAGTTTGAAA	GTTTACCAAT	GGCTATATA	TAAATCAACT	TGATAATATA	AAAAAAATTT
150	160	170	180	190	200	210
CAATTGAAA	AGGGGCTAAA	ATATTCTCAA	AGTATTGGAA	ATGCTACAAA	ACTACCATCC	GTCACCTAT
220	230	240	250	260	270	280
TGACTGCAAA	ATAAAATTAT	TATCCACCTT	TGAGTTTAAA	ATTGACTACT	TATATAACAA	TTCTAAATTT
290	300	310	320	330	340	350
AAACTATTTT	AATACTTTAA	AAAATACATG	GGTTTCAAAI	ATTTAATATA	ATTTAATTTA	TGAATATCAT
360	370	380	390	400	410	420
TTATAAACCA	ACCAACTACC	AAGTCATTAA	TCATTAAATC	CCACCCAAAT	TCTACTATCA	AAATTGTCTT
430	440	450	460	470	480	490
AAACACTACT	AAAACAAGAC	GAATTGTTC	GAGTCCGAAT	CGAAGCACCA	ATCTAATTTA	GGTTGAGCCC
500	510	520	530	540	550	560
CATATTTAGG	ACGACACTTT	CAATAGTATT	TTTTTCAAGC	ATGAATTGTA	AAITTAAGAT	TAATGGTAAA
570	580	590	600	610	620	630
GAAAGTAGAC	ATCCCGAATT	AATTCATGCC	TTTTTTAAAT	ATAATTATAT	AAATATTTAT	CATTIGTTTT
640	650	660	670	680	690	700
AAATATTAAA	ACTTGAATAT	ATTATTTTTT	TAAAAATTAT	CTATTAACTA	CCATCAGATA	ATTGAGACTA
710	720	730	740	750	760	770
AGGAATAATT	AAGATGAACA	TAGTGTTTAA	TTAGTAATCG	ATCGGTAGTA	AAITTTATTA	TAAATTATAT
780	790	800	810	820	830	840
CAATAAGTTA	AATTATAACA	AATATTTGAG	CGCCATGTAT	TTAAAAAAT	ATTAAATAGT	TTCAATTTAA
850	860	870	880	890	900	910
AACCGTTACA	TAAATGCTCA	ATTTTGAACC	CAAAAGTCCA	TCAGAAGGGT	ATTTTAGAGC	CAATAGGGGG
920	930	940	950	960	970	980
ATGAGAAGCA	TATTTTCAAG	CCAATATGTC	ATGGATCAAG	GATAATTTTG	TATCATTTCT	AATACTTTAA
990	1000	1010	1020	1030	1040	1050
ACATATTTTA	GCTCATTTTC	CCTTCTTTAG	TTTAAGAGCT	ATACTGTTAG	TTTATCGAAT	ATCATCTATT
1060	1070	1080	1090	1100	1110	1120
ATTTCGGTCT	TAAATATTTT	TTTATTTTAT	AAATTTTTTA	AAAAATAATT	ATTTTTTCCA	TTTAAGTTTG
1130	1140	1150	1160	1170	1180	1190
ATTGTAAATTA	ATTTTAAAAA	ATTACCAACA	TATAAATAAA	ATTAAATATT	AACAAACAAT	TGTAACATTA
1200	1210	1220	1230	1240	1250	1260
TATTTTAAAA	ATTATTCAAA	ATAAATATTT	TAAACATCA	TATAAAGAA	ATACGACAAA	AAAAATGAGA
1270	1280	1290	1300	1310	1320	1330
CGGGACAAGA	CAAGCCAGAC	AAAAATGTC	AAGAAACTCT	TTCTCTTAAA	TATCTCTCAT	CCAAAGTAAT
1340	1350	1360	1370	1380	1390	1400
ATAATACCCA	TTATAATTAA	CCATATTGAC	CAACTCAAAC	CGCTTAAAT	GTATAAATAG	ACAAAGCCTT
1410	1420	1430	1440	1450	1460	1470
CCCATACCTC	TTATCATAAA	AAAAATAATA	ATCTTTTTCA	ATAGACAAGT	TTAAAAACCA	TACCATATAA

FIGURE 5

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1480	1490	1500	1510	1520	1530	1540
CAATATATCA	TGGTATATCCA	AAGGAATACT	ATTCTCCTTC	TCATTATTAT	TTTTGCTTCA	TCAATTTCAA
1550	1560	1570	1580	1590	1600	1610
CTTGTAGAAG	CAATGTTATT	GATGACAAAT	TATTCAAACA	AGTTTATGAT	AATATTCTTG	AACAAGAAAT
1620	1630	1640	1650	1660	1670	1680
TGCTCATGAT	TTTCAAGCTT	ATCTTCTTAA	TTTGAGCAAA	AATATTGAAA	GCAACAATAA	TATTGACAAG
1690	1700	1710	1720	1730	1740	1750
GTTGATAAAA	ATGGGATTAA	AGTGATTAA	CTACTTACCT	TTGGAGCTAA	GGGTGATGGA	AAAACATATC
1760	1770	1780	1790	1800	1810	1820
ATAATATTCT	AAGTATTTAA	ATATTGGAAT	ATATTCTTGG	GGATGAAAAT	GATAGAGAAT	ATAAGAATTA
1830	1840	1850	1860	1870	1880	1890
TTTGGAAAGGA	TGAAAAGTTA	TATTTTATAA	AGTAGAAAAT	TATTTTCTCG	TTTTTAGTAA	TTAAAGGTGA
1900	1910	1920	1930	1940	1950	1960
AAATGAGTT	TTCTCGTAAG	CGAGGAAAGT	CATTTTCCAT	GGAAGTGTAT	TTTTTTTTTA	CTTTTAATAA
1970	1980	1990	2000	2010	2020	2030
CGTCATAGTA	TTTGCTATAC	TCAAGAATAA	GACACTAATA	TTGATGTTTA	GTGCTCGAAA	AGAAATTGAT
2040	2050	2060	2070	2080	2090	2100
AGTAATTTTG	CTAATATAAC	TATCAATTTC	TTATATGTAT	ATTTTTC AAC	CAAAATAACA	AAGCCTAATC
2110	2120	2130	2140	2150	2160	2170
CAATAAGTGG	GCCTCTAGAA	TAAAGAGTAA	GTTCTATTAA	TTCTTAACCT	TATTTAATTT	TATGGAAACC
2180	2190	2200				
TCGACAAAAC	GACAATGCTC	AACTTATATT	CGAATTC			

FIGURE 5

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (4): C07H 15/12 C12N 15/00 C12N 5/00 A01H 1/04 U.S. CL: 536/27 435/172.3 435/320 435/240.4 800/1		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	435/172.3, 240.4, 320 536/27 800/1	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	Plant Physiology, Volume 83, issued April 1987, (Rockville, Maryland, USA), Boston et al., "Expression from heterologous promoters in electroporated carrot protoplasts", pages 742-746, see pages 742-743 in particular.	1-3, 6, 9, 12, 13, 16, 17 8, 14, 18-24
Y	Molecular and General Genetics, Volume 200, issued August 1985, (Heidelberg, Germany), Mansson et al., "Characterization of fruit specific cDNAs from tomato", pages 356-361, see pages 356, 358 and 360 in particular.	1-6, 10, 14, 15, 18-24
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
23 JULY 1988		07 SEP 1988
International Searching Authority		Signature of Authorized Officer
ISA/US		DAVID T. FOX <i>David T. Fox</i>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<u>Bio/Technology</u> , Volume 3, issued March 1985, (New York, New York, USA), Facciotti et al., "Light-inducible expression of a chimeric gene in soybean tissue transformed with Agrobacterium," pages 241-246, see page 241 in particular.	1-3,8,9, 11,14, 18-24
Y,P	<u>Molecular and General Genetics</u> , Volume 208, issued June 1987, (Heidelberg, Germany), Sheehy et al., "Molecular characterization of tomato fruit polygalacturonase", pages 30-36, see pages 30 and 33 in particular.	7,14,18, 23
Y	<u>Proceedings of the National Academy of Sciences USA</u> , Volume 83, issued September 1986, (Washington, D.C., USA), Della Penna et al., "Molecular cloning of tomato fruit polygalacturonase: analysis of polygalacturonase mRNA levels during ripening," pages 6420-6424, see page 6422 in particular.	7,14,18, 23
Y	<u>Nucleic Acids Research</u> , Volume 14, issued November 1986, (Oxford, England), Grierson et al., "Sequencing and identification of a cDNA clone for tomato polygalacturonase," pages 8595-8603, see pages 8598-8599 in particular.	7,14,18, 23
Y	<u>Proceedings of the National Academy of Sciences USA</u> , Volume 83, issued August 1986, (Washington, D.C., USA), Ecker et al., "Inhibition of gene expression in plant cells by expression of antisense RNA," pages 5372-5376, see page 5373 in particular.	5,7,14, 23

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Proceedings of the National Academy of Sciences USA, Volume 82, issued May 1985, (Washington, D.C., U.S.A.), Sengupta-Gopalan et al., "Developmentally regulated expression of the bean beta-phaseolin gene in tobacco seed," pages 3320-3324, see page 3321 in particular.

1-3,8,9,
11,14,
18-24

V. ☐ **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out. Specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²**

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.